INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 6, p. 216-222, 2017

RESEARCH PAPER OPEN ACCESS

Prevalence of multidrug resistant bacteria in Rawal lake and its catchment area-an alarming situation for Rawalpindi city

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Key words: *E. coli, S. typhi, Klebsiella,* Waste water, Antibiotics

http://dx.doi.org/10.12692/ijb/10.6.216-222 Article published on June 30, 2017

Abstract

Water samples were taken from Rawal Lake and its catchment area during January and August to study seasonal impact on prevalence of antibiotic resistant bacteria namely *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*) and *Klebsiellap neumonia* (*Klebsiella*) in the aquatic environment. The Rawal Lake and its whole catchment area were contaminated with the pathogenic bacteria except the two tributaries away from human population and activities. Results of the investigation revealed that isolates of *E. coli*, *S. typhi* and *Klebsiella* collected during August and January were 45 to 100% and 40 to 90% found antibiotic resistant respectively. This difference may be due season depended human activities and favorable temperature. Selected isolates of the three bacteria were examined for Erythromycin (Ert), Kanamycin (Kan), Ciprofloxacin (Cip), Norfloxacin (Nor), Ofloxacin (Oxl), Ampicillin (Amp), Vancomycin (Van), Amoxicillin (Amx). No isolate was resistant to less than four antibiotics understudy, highest resistance was shown by isolate of *E. coli* named as EC6 which was resistant to all the eight antibiotics. Untreated wastewater from recreational sites, hotels and hospital entering into the catchment area was mainly observed responsible for the resistant bacteria.

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Introduction

Treatment of infectious diseases is continuously improving from the last 60 years, which result in reduction of the morbidity and mortality. This has been done due to our better knowledge about diseases and mainly because of rapid development of effective and safe antibiotics. The safe antibiotics have been able to attack specific disease causing organism. Initial antibiotics (sulfonamides and penicillin) were not available to public and were reserved for use by army during World War II (Alanis, 2005). During the last many years, production of pharmaceuticals has rapidly increased. Approximately 3000 compounds are in use as medicine and annual production reached up to hundreds of tons (Sim *et al.* 2011). It was estimated that worldwide annual consumption of antibiotics is about 100,000 to 200,000 tons (Zheng *et al.*, 2011). There are about 350 licensed pharmaceutical manufacturers in Pakistan. Annual pharmaceutical market in Pakistan is over $ 1.25 billion (Medinet, 2013). Share of antibiotics in the market was approximately $ 140 million during the year 2007 (Shoaib, 2007). Antibiotics like ß-lactams, streptomycins and others are produced by soil bacteria in natural environment. But concentration of the antibiotics remains very low or usually below the detection limits (Kummerer, 2009a). On the other hand, release of antibiotics from anthropogenic sources can be up to several mg L^{-1} (Larsson *et al.*, 2007). Common sources responsible for the presence of pharmaceutical compounds in the environment are hospitals, municipal waste water, livestock farms and pharmaceutical manufacturing units (Zhang *et al.*, 2008). Most of the antibiotics are not completely metabolized in human and animals bodies and about 25% to 75% of antibiotics excrete into the environment in active form through urine and feces. So antibiotics used by humans and animals can be found at various concentrations in hospital effluents, municipal wastewater and discharge of wastewater treatment plants (Zheng *et al.*, 2011).

As a result receiving water bodies like rivers become contaminated with the antibiotics and the compounds become the part of food chain (Jones-Lepp *et al.*, 2012).

Presence of antibiotics in the environment causes selective pressure on bacterial populations, which lead to resistant bacteria even at low concentrations (Stepanauskas *et al.*, 2006). Antibiotic resistant strains belong to pathogenic bacteria can be found from aquatic environment and because of long time exposure to different clinical antibiotics they are resistant to more than one clinical antibiotics (Munir *et al.*, 2011). The problem of antibiotic resistant bacteria and their resistant genes is becoming a major global health issue because antibiotic resistant bacteria can distribute resistant genes among microbial community (Schmieder and Edwards. 2012). Research is underway to solve the problem by developing new antibiotics. However, soon the bacteria started acquiring resistance to the newly developed drugs and begin to survive as multi drug resistant organism (Hori *et al.*, 1993).

Outbreak of waterborne diseases in developing countries is a common phenomenon and it also takes place in developed countries because of close interaction of natural water resources and manmade water distribution system (Blasco *et al.*, 2008). Therefore, the aim of the study to investigate the multi antibiotic resistance in common pathogenic bacteria namely *E. coli*, *S. typhi* and *Klebsiella* in Rawal lake and its catchment area which is common source of drinking water for Rawalpindi city of Pakistan.

Materials and methods

Study Area

Samples were taken from selected tributaries and sites of Korang River (catchment area of Rawal Lake). Rawal Lake is main source of drinking water for Rawalpindi city. Korang River is receiving untreated wastewater from different areas of Murree (popular recreational site) and some areas from Islamabad (capital city of Pakistan). No wastewater treatment facility available prior to final disposal of the municipal wastewater into the River.

Isolation and Identification of E. coli, Salmonila Typhiand Klebsiella

For isolation and initial identification of *E. coli*, *S. typhi* and *Klebsiella* the water samples were diluted up to 10^2 to 10^4 times by serial dilution method.

From prepared samples 0.1 ml was transferred to three types of petriplates containing Brilliance *E. coli*/coliform selective agar and Brilliance *Salmonella* agar and HiCome™ *Klebsiella* Selective agar for isolation of *E. coli* and *S. typhi and Klebsiella* respectively. To spread the sample properly on selective growth media, the plates were rotated clockwise and anti-clockwise and by using (sterilized) L-shaped cell spreader. All the three plates were incubated at 37 °C for 24 h. In the petriplates containing Brilliance *E. coli*/coliform selective agar, number of purple colored *E. coli* colonies appeared *S. typhimurium* on Brilliance *Salmonella* agar were also appeared as purple colored colonies while *Klebciella* appeared in purple-magenta coloured colonies (Clark *et al.*, 1958; Koczura *et al.*, 2012). For conformation, 16srRNA was performed (of some selected isolates) by Macrogen (Macrogen Inc., Geunchen-gu South Korea) using universal forward (5´GGATTAGATACC CTGGTA3´) and reverse primer (5´CCGTCAATTCM TTTRAGTTT3´).

Antibiotic sensitivity test

Different concentrations of antibiotics were prepared by using Iso-sensitest broth. One ml (10^3 CFU/ml) of each bacterium inoculum was transfer to each concentration. The cultures were incubated at 37 °C for 24 h. Optical density (OD) of the culture was measured by adjusting the wavelength at 600nm after 0 and 24 h. Values of minimum inhibitory concentration (MIC_{90}) for the three bacteria according to specific antibiotics were used to study the tolerance level of the pathogens. The isolates which were able to increase cell population in antibiotic concentrations equal to or higher than the MIC_{90} were considered to be resistant (Roychoudhury *et al.*, 2001; Huang *et al.*, 2012). Each experiment was run in duplicate.

Results and discussion

The bacterial samples from the selected sites (from catchment area of Rawal Lake) were collected during January and August. All the three bacteria under study were detected from the water samples, except one sample that was collected from tributary with no population on its catchment area.

It is because main source of antibiotic resistant bacteria are hospitals, human population and commercial activities (Scott *et al.*, 2015; Maqsood *et al.*, 2014). The isolates of *E. coli* collected from main stream of Korang River were found more resistant as compare to the tributary isolates. The isolates of *E. coli* collected during August were 70% to 95% resistant to antibiotics, while the isolates of *E. coli* collected during December were 40% to 90% resistant (Fig. 1).

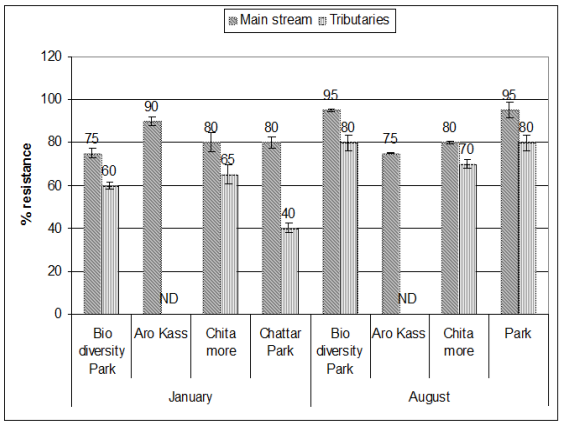

Fig. 1. Percent resistance of *E. coli* isolates (n=20 for each sampling site) isolated during the month of January and August from catchment area of Rawal Lake.

Same trend was observed in case of *S. typhi* and *Klebsiella* i.e. 70% to 95% and 65% to 90% isolates collected during January and 90% to 100% and 45% to 90% isolates sampled during August were found antibiotic resistant respectively (Fig. 2 and 3). Similar trend was shown by isolates of *E. coli*, *S. typhi* and *Klebsiella* isolated from water samples of Rawal Lake during August were more resistant as compare to isolates isolated during January (Fig. 4). Epidemics of water borne diseases in the study area usually erupt during summer (May to September), this was the reason that more resistant bacteria detected during August instead of January, because increase in antibiotic concentration and antibiotic resistant bacteria in water bodies usually depend upon season and epidemics (Lucia *et al.*, 2014) and summer temperature also support the growth of bacteria. Untreated municipal wastewater from local population, hospitals, institutes etc. established in

nearby areas of the Rawal Lake also contribute to enhance the issue; because water bodies receiving untreated hospital and municipal waste are contaminated with higher concentration of antibiotics and more antibiotic resistant bacteria as compare to water bodies without the point sources (Hocquet *et al.*, 2016; Ahmad *et al.*, 2013).

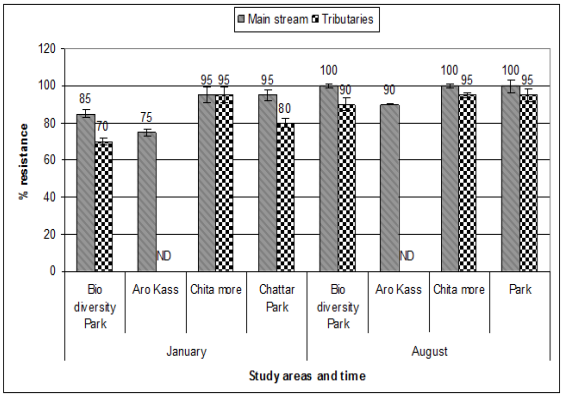

Fig. 2. Percent resistance of isolates (n=20 for each sampling site) of *S. typhi* isolated during the month of January and August from catchment area of Rawal Lake.

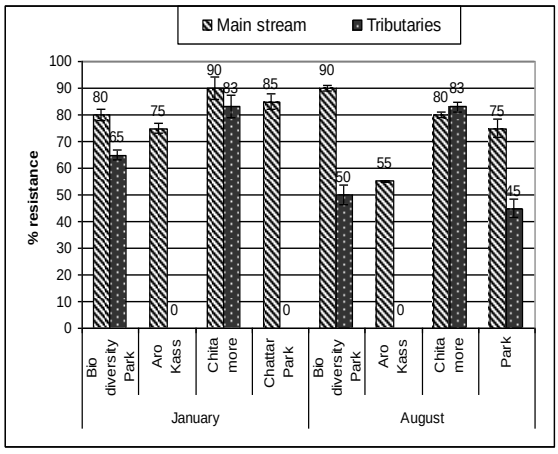

Fig. 3. Percent antibiotic resistance of *Klebsiella* (n=20 for each sampling site) isolated during the month of January and August from catchment area of Rawal Lake.

Selected isolates of *E. coli*, *S. typhi* and *Klebsiella* on the bases of growth response under the stressed environment of antibiotics were selected for multidrug resistance analysis. The selected isolates were tested against eight antibiotics including Erythromycin (Ert), Kanamycin (Kan), Ciprofloxacin (Cip), Norfloxacin (Nor), Ofloxacin (Oxl), Ampicillin (Amp), Vancomycin (Van) and Amoxicillin (Amx). Nine isolates of *E. colli* were examined for multi antibiotic resistance; EC3 was found resistant to only four antibiotics. Other than EC3 all *E. colli* isolates were resistant to more than four out of the eight. While EC6 was proved to be resistant to all eight antibiotics understudy.

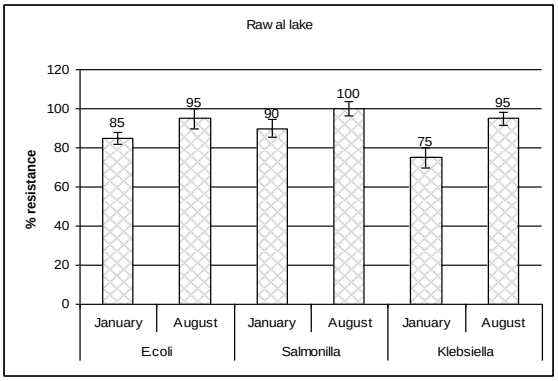

Fig. 4. Percent resistance of *E. coli*, *S. typhi* and *Klebsiella* isolated from Rawal Lake during January and August (n=20 for each case).

The results of fourteen isolates of *S. typhi* illustrate that five out of fourteen were resistant to seven and no isolate was resistant to less than four antibiotics under study. Among the seven isolates of *Klebsiella,* all are resistant to at least four antibiotic while KS2 was resistant to seven antibiotics under study (Table 1). Usually long term exposure to antibiotics at low concentration present in aquatic environment causes development of multidrug resistance, and multidrug resistance can also be develop by getting antibiotic resistance gene from the environment (Fumie *et al.*, 2013). In short, it can be say that bacteria with intrinsic antibiotic resistance can get antibiotic resistant genes from environment and can act as reservoir of multidrug resistant gene for next generations and also for environment (Ash *et al.*, 2002). It's a nonstop phenomenon to develop resistant against new antibiotics after exposure. The situation is worrisome in developing countries because of poverty, malnutrition, unavailability of clean water and lack of health facilities (Alvan *et al.*, 2011) providing base to develop resistant pathogenic bacteria.

Table 1. Summary of antibiotic resistance of selected isolates of *E. coli*., *S. Typhi*., and Klebsiella against Erythromycin (Ert), Kanamycin (Kan), Ciprofloxacin (Cip), Norfloxacin (Nor), Ofloxacin (Oxl), Ampicillin (Amp), Vancomycin (Van), Amoxicillin (Amx).

No.		Antibiotic resistant to
Isolates of *Escherichia colli*		
1	EC1	Ert, Kan, Cip, Amp, Van, Amx
2	EC2	Ert, Kan, Amp, Oxl, Van, Amx
3	EC3	Ert, Kan, Cip, Amp
4	EC4	Ert, Kan, Cip, Nor, Oxl, Amp, Amx
5	EC5	Ert, Kan, Amp, Van, Amx
6	EC6	Ert, Kan, Cip, Nor, Oxl, Amp, Van Amx
7	EC7	Ert, Cip, Nor, Oxl, Van, Amx
8	EC8	Ert, Kan, Cip, Nor, Amp, Amx
9	EC9	Ert, Kan, Nor, Oxl, Amp, Van, Amx
Isolates of *Salmonilla typhy*		
1	ST1	Ert, Cip, Amp, Van, Amx
2	ST2	Ert, Cip, Nor, Amp, Van, Amx
3	ST3	Kan,Cip,Amp,Van, Amx
4	ST4	Ert,Kan,Cip,Amp,Van, Amx
5	ST5	Ert, Kan,Cip, Nor, Oxl, Amp, Amx
6	ST6	Ert, Kan, Cip, Nor, Amp, Van, Amx
7	ST7	Ert, Kan, Cip, Nor, Amp,Van,Amx
8	ST8	Kan, Cip, Amp, van, Amx
9	ST9	Ert, Kan, Cip, Nor, Oxl, Amp, Amx
10	ST10	Ert, Kan, Cip, Nor, Amp, Amx
12	ST11	Ert, Kan, Amp, Amx
13	ST12	Ert, Kan, Cip, Nor, Amp, Van, Amx
14	ST13	Erth, Kan, Amp, Van, Amx
Isolates of *Klebsiella*		
1	KS1	Ert, Kan, Cip, Amp, Van, Amx
2	KS2	Ety, Kan, Cip, Nor, Amp, Van,Amx
3	KS3	Kan, Cip, Amp, Amx
4	KS4	Ert, Cip, Amp, Van
5	KS5	Ert, Kan, Cip, Amp, Van, Amx
6	KS6	Kan, Oxl, Amp, Van, Amx
7	KS7	Kan, Cip, Olx, Amp, Van

Conclusion

The results of the study indicates that almost all the catchment area of the Rawal lake except the tributaries not contaminated by human activities, the main source of drinking water for Rawalpindi city is contaminated with pathogenic bacteria. About 40% to 100% isolates of the three types of bacteria were found antibiotic resistant. It was also examined that most of antibiotics under study were not effective at their described MIC values because of development of resistance against the antibiotics due to environmental exposure.

Acknowledgements

Authors would like to pay thanks to Environmental Protection Agency Punjab and Biotroll Private Limited Lahore and Holy Family Hospital Rawalpindi to provide Laboratory and technical assistance during the research work.

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